Davis, Minh-Tam

From: Chan, Christina
Sent: Monday, November 06, 2006 5:35 PM
To: Davis, Minh-Tam; STIC-Biotech/ChemLib
Subject: RE: Rush search request for 09/856812

Please rush. Thanks Chris

Chris Chan
TC 1600 New Hire Training Coordinator and SPE 1644
(571)-272-0841
Remsen, 3E89

-----Original Message-----
From: Davis, Minh-Tam
Sent: Monday, November 06, 2006 5:35 PM
To: Chan, Christina
Subject: Rush search request for 09/856812

Please search in commercial database, issued patent files, and pGPUB:
The peptide SEQ ID NO:42, **without size limitation** for the sequences in the database.
Thank you.
MINH TAM DAVIS
ART UNIT 1642, ROOM 3A24, MB 3C18

Results can be found in SCORE.

NPL_√_ ADONIS √_ MIC___ BioT_√_Main__NO__Vol NO__ NOS___CKCite__Dup__Int_W/C

~~STIC-ILL~~ RC 3821.A1 I57 11/7

From: Davis, Minh-Tam
Sent: Monday, November 06, 2006 5:35 PM
To: STIC-ILL
Subject: Reprint request for 09/856812

7944517 Genuine Article#: 227FU Number of References: 20
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Author(s): Rimoldi D (REPRINT) ; Salvi S; Reed D; Coulie P; Jongeneel VC; DePlaen E; Brasseur F; Rodriguez AM; Boon T; Cerottini JC
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Journal: INTERNATIONAL JOURNAL OF CANCER, 1999, V82, N6 (SEP 9), P901-907
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Thank you.
MINH TAM DAVIS
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Int. J. Cancer: 82, 901–907 (1999)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

cDNA AND PROTEIN CHARACTERIZATION OF HUMAN *MAGE-10*

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MAGE genes are frequently expressed in several types of human malignancy and code for antigens recognized by cytotoxic T lymphocytes. We have previously described a monoclonal antibody (MAb), named 6C1, that recognizes the MAGE-1 protein and cross-reacts with a 72-kDa protein present in lysates of melanoma cells such as MZ2-MEL. To identify this protein, we have screened an expression library prepared from MZ2-MEL cells. Several clones that encoded a protein recognized by antibody 6C1 contained a sequence identical to that of _MAGE-10_, another member of the _MAGE-A_ gene family. Full-length _MAGE-10_ cDNA clones, obtained after screening additional cDNA melanoma libraries, were found to be approximately 2.5 kb in length. _In vitro_ translation and transient transfection experiments indicated that _MAGE-10_ codes for a protein of approximately 72 kDa. This product was recognized by MAb 6C1 as well as by a polyclonal serum raised against a MAGE-10 peptide, thus demonstrating its identity with MAGE-10. Analysis of MAGE-10 mRNA by RT-PCR confirmed its presence in testis and placenta but not in other normal tissues. Expression of MAGE-10 in melanoma tumors was found to parallel that of MAGE-1. Western blot analysis with the polyclonal anti-MAGE-10 antibody showed the presence of MAGE-10 in lysates of purified trophoblast cells. Immuno-cytochemistry of cultured melanoma cells indicated that MAGE-10 is a nuclear protein. _Int. J. Cancer_ 82:901–907, 1999.
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MAGE-1 is the first gene found to code for a human tumor antigen recognized by autologous cytolytic T lymphocytes (CTLs) (reviewed by Romero, 1996; Van den Eynde and Boon, 1997). *MAGE-1* belongs to the *MAGE-A* gene family that consists of 12 homologous genes (*MAGE-1* to *-12*) located in the q28 region of chromosome X (De Plaen *et al.*, 1994; Rogner *et al.*, 1995). A separate cluster of *MAGE* genes is located on Xp21 (*MAGE-B* family) (Muscatelli *et al.*, 1995). A new gene, *MAGE-C1*, sharing significant homology with other *MAGE* genes, has been localized on Xq26 (Lucas *et al.*, 1998). *MAGE-A* genes are expressed by a variety of human cancers, such as melanoma, breast and bladder carcinoma or non-small-cell lung cancer. Among the different family members, *MAGE-1, -2, -3, -4, -6* and *–12* are expressed more abundantly in tumor tissues and cell lines. None of these genes appears to be transcribed in normal adult tissues, with the exception of testis. *MAGE-3, -4, -8, -9, -10* and *-11* transcripts have also been detected in placenta (De Plaen *et al.*, 1994). Sequencing of *MAGE-1, -2, -3, -4, -6* and *-12* outlined a conserved genomic structure with a minimum of 2 exons, the last one being the longest and containing the coding region. MAGE-A proteins consist of 309 to 319 amino acids. Their function is still unknown.

Tumor-specific CTLs recognizing MAGE-1 and -3 have been isolated from cancer patients. In addition, several CTL epitopes derived from MAGE-1, -2 and -3 are capable of eliciting CTL responses *in vitro* (reviewed by Romero, 1996; Visseren *et al.*, 1997). Thus, because of the potential use of *MAGE* gene products as targets for specific immunotherapy, expression of *MAGE* genes has been widely studied in tumors, mainly by RT-PCR analysis. In addition, antibodies against MAGE recombinant proteins have been produced to investigate expression of the genes at the protein level. Using such reagents, MAGE-1, -3, -4 and -11 have been identified as 45- to 50-kDa proteins (Kocher *et al.*, 1995; Amar-Costesec *et al.*, 1994; Shichijo *et al.*, 1995; Jurk *et al.*, 1998).

We have reported the generation of MAbs against a recombinant MAGE-1 protein (Carrel *et al.*, 1996). These antibodies recognize, in addition to MAGE-1, a product of approximately 72 kDa. This protein exhibits regulation of expression similar to that of *MAGE-1* as it is co-expressed with the latter in a series of melanoma cell lines and its expression is induced by hypomethylating agents. We have now cloned the cDNA of this 72-kDa protein and report here its identification with the product of the *MAGE-10* gene.

MATERIAL AND METHODS

Screening of a melanoma expression library

An expression library was prepared from the MZ2-MEL-43 melanoma cell line using the Superscript Lambda System (GIBCO BRL, Gaithersburg, MD). Bacteriophages (4×10^5 pfu) were plated and transferred to Hybond-C nitrocellulose filters (Amersham, Aylesbury, UK). Filters were incubated with monoclonal antibody (MAb) 6C1 (hybridoma supernatant diluted 1:4 in RPMI, 10% FCS) after blocking overnight with 5% milk powder in PBS. After washing with PBS, 0.5% Tween-20, filters were incubated with horseradish peroxidase–conjugated sheep anti-mouse Ig (Amersham, 1:3,000 dilution in PBS, 5% milk powder). After washing with PBS, 0.5% Tween-20, signals were detected using an ECL system (Amersham). Positive plaques were subjected to secondary and tertiary screening. Phage inserts were then amplified by PCR using the primers 5'-GTGGCGACGACTCCTGGAG-3' (λgt 22 primer 1) and 5'-CAGACCAACTGGTAATGGTAGCG-3' (λgt 22 primer 2) and the following cycling parameters: 1 min at 94°C, 1 min at 61°C and 1 min at 72°C for 30 cycles and a final extension at 72°C for 10 min. A partial 5' sequence of the clones was obtained by directly sequencing with a Sequenase version 2.0 kit (USB, Cleveland, OH) using the λgt 22 primer 1. The insert from one of the *MAGE-10* clones, 13.1, was subcloned into Bluescript plasmid and the entire sequence obtained by automatic sequencing from both directions using T3 and T7 primers. The insert was confirmed to be a partial *MAGE-10* cDNA of 1,400 bp, starting at position 2770 (according to the numeration of the previously published genomic sequence, HSU10685) and extending an extra 660 bp at the 3' end.

Sequence homology searches in GenBank were performed using the Blast program of the National Center for Biotechnology Information server.

Full-length cDNA cloning

A 485-bp Hpa I fragment (corresponding to nucleotides 1138–1623 of sequence HSU10685) isolated from the 1.4-kb *MAGE-10* insert from phage 13.1 was ^{32}P-labeled using a random priming DNA-labeling kit (Boehringer-Mannheim, Mannheim, Germany)

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and used to screen 2 libraries from the melanoma cell line LB373-MEL-4.0 in pcDNA-I/Amp and pCEP-4 (Lucas *et al.*, 1998). Hybridizations of Hybond-N filters (Amersham) were performed in 5× SSC, 5× Denhardt's, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA at 65°C overnight. Filters were then washed 3 times for 10 min at room temperature with 1× SSC, 0.1% SDS; once for 20 min at 65°C with 1× SSC, 0.1% SDS; and twice for 20 min at 65°C with 0.1× SSC, 0.1% SDS. Inserts of positive clones were automatically sequenced using T7 and SP6 primers or a pCEP-4 forward primer (Invitrogen, La Jolla, CA) for pcDNA-I/Amp or pCEP-4 plasmids, respectively. All sequences appeared to be identical, except for 5' endings, which showed truncated versions of exon 1, the longest one being of 111 bp.

In vitro transcription/translation of MAGE-10 cDNA

MAGE-10 clones isolated from the pcDNA-I/Amp library were used for *in vitro* transcription/translation using a TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Each DNA (1 μg) was translated with a T7 RNA polymerase in the presence or absence of ^{35}S-labeled methionine, according to the manufacturer's instructions. A luciferase control plasmid, supplied in the kit, was used as a positive control and water as a negative control in place of DNA. Translation products were subjected to PAGE under reducing conditions.

Cell culture and transfection

Melanoma cell lines and 293T cells were grown in RPMI 1640, 10% FCS and DMEM, 10% FCS, respectively. 293T cells were transfected by the Ca–phosphate technique (Ausubel *et al.*, 1998). Cells were collected and lysed in a Nonidet/P40 buffer 48 hr after transfection.

Western blot analysis

Western blot analyses were performed as described (Carrel *et al.*, 1996) using MAb 6C1 as undiluted hybridoma supernatant or the anti-MAGE-10 rabbit polyclonal serum (1:5,000 dilution). Anti-chorionic gonadotropin β polyclonal antibody was obtained from Anawa (Wangen, Switzerland). Peroxidase-conjugated anti-rabbit and anti-mouse Ig secondary antibodies and the ECL detection system were from Amersham.

Fluorescent immuno-histochemistry

Cells were cultured in glass multichamber slides (Lab-Tek, GIBCO) and fixed in acetone for 5 min at –20°C. Slides were incubated with primary antibodies (undiluted hybridoma supernatants or 1:2,000 diluted rabbit sera) followed by Cy3-conjugated sheep anti-mouse or donkey anti-rabbit IgG (Jackson ImmunoResearch; Dianova, Hamburg, Germany). Nucleic acid staining was performed with YOYO-3 iodide (1:2,000 dilution in PBS; Molecular Probes, Leiden, the Netherlands) for 5 min at room temperature before mounting. Pictures were obtained with a Photonic Science camera mounted on a Zeiss Axioskop microscope.

Preparation of anti-MAGE-10 serum

A polyclonal rabbit serum was raised against a synthetic peptide corresponding to the C terminus of the predicted MAGE-10 sequence (QDRIATTDDTTAMASASSSATGSFSYPE). This region was chosen to avoid cross-reactivities with other MAGE proteins as it extends beyond the conserved sequence common to the other members of the MAGE family (De Plaen *et al.*, 1994). A second peptide, consisting of the P30 tetanus toxin T-cell epitope sequence (Valmori *et al.*, 1992) followed by the MAGE-10 sequence indicated above, was synthesized, and the 2 peptides were used in an alternate fashion to immunize a rabbit. Injections and bleedings were performed at Eurogentec (Seraing, Belgium). The immune serum was tested by an ELISA against the immunizing and control peptides. Peptide-blocking experiments were performed to check the specificity of the signal, and pre-immune serum was always used as a negative control in the different experiments.

PCR and Northern blot analyses

RNA extractions and cDNA synthesis were performed as described by Brasseur *et al.* (1995). For some tissue samples, random priming was used for cDNA synthesis in parallel to oligo-dT priming to control for potential partial RNA degradation. PCR to amplify MAGE-10 from cDNA was performed with primers 5'-GGAACCCCTCTTTTCTACAGAC-3' (M10–3, upper) and 5'-TCCTCTGGGGTGCTTGGTATTA-3' (M10–4, lower), located in the 2nd and 4th exon, respectively, with the following cycling parameters: 1 min at 94°C, 30 sec at 55°C and 1 min at 72°C for 30 cycles and a final extension at 72°C for 10 min. To verify the sequence of the amplified fragments, separated products were purified from gel using a GeneClean kit (BIO 101, La Jolla, CA) and sequenced using a ThermoSequenase cycle sequencing kit (Amersham). PCR for detection of *MAGE-1* and β-actin were as described by Brasseur *et al.* (1995). To obtain the complete sequence of intron 1, a *MAGE-10*-containing cosmid was amplified using a forward primer located in the 1st exon (5'-CGGGACTCGGGGATCAGAGA-3') and a reverse primer in the known part of intron 1 (5'-TCCCTCCTGCTGAATCGTGTAT-3'). The amplified product was subcloned into pCR 2.1 using a TA cloning kit (Invitrogen) and subjected to automated sequencing. Electrophoresis of RNA in formaldehyde/agarose gel and Northern blotting was according to standard procedures (Ausubel *et al.*, 1998). As a

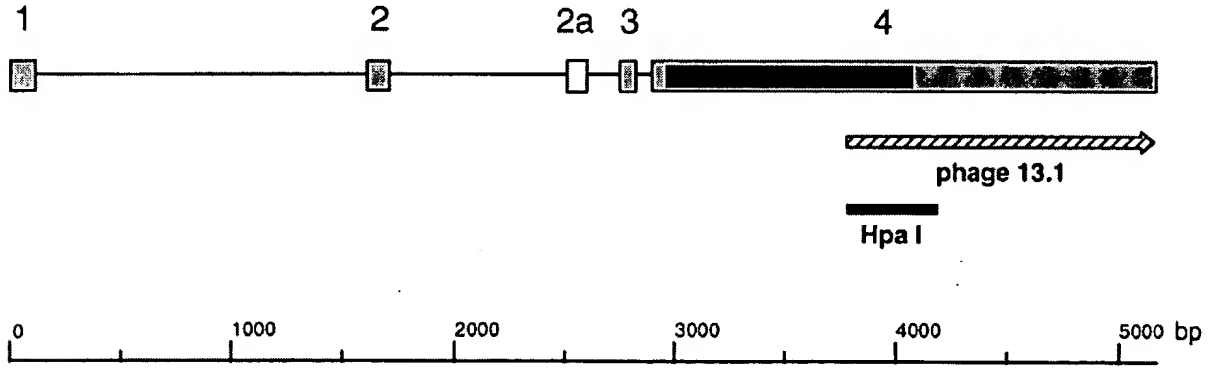

FIGURE 1 – Genomic structure of *MAGE-10*. This structure was generated by comparing sequences of several cDNA clones with the known genomic *MAGE-10* sequence (HSU10685). The complete sequence of intron 1 was obtained by PCR amplification on a cosmid clone. Shaded boxes represent exons, and the coding region is marked in black. The alternatively spliced exon 2a is indicated as an open box. The arrow indicates the sequence of phage 13.1 obtained by screening the expression library with MAb 6C1. Below is the Hpa I fragment used for screening the LB373-MEL-4.0 libraries.

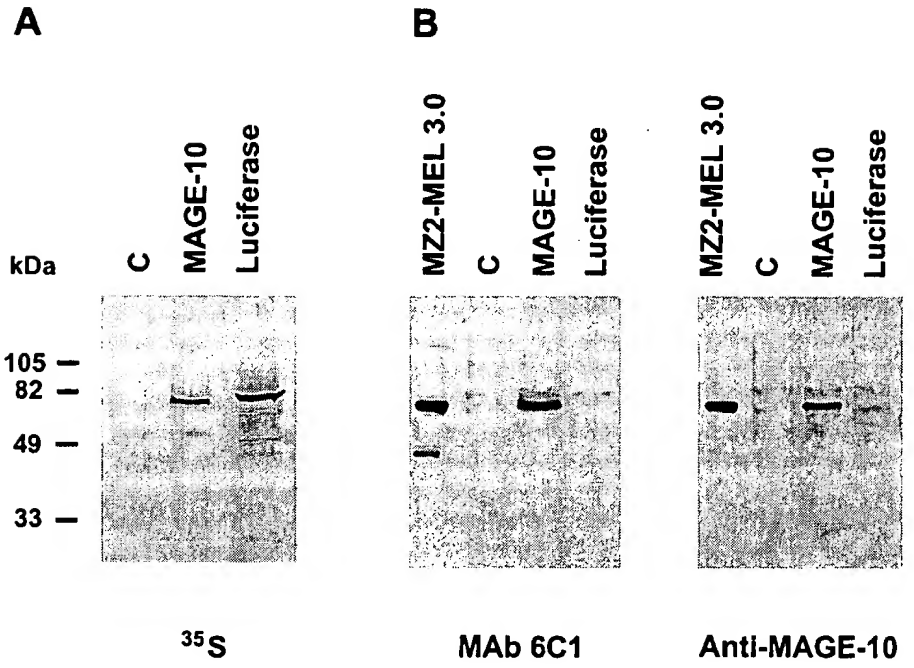

FIGURE 2 – *In vitro* transcription and translation of a full-length *MAGE-10* cDNA. A full-length cDNA clone was transcribed and translated *in vitro*. A luciferase plasmid and water were included as positive and negative (C) control, respectively. *(a)* Translation was carried out in the presence of ^{35}S-labeled methionine, and the products were analyzed by SDS-PAGE under reducing conditions followed by autoradiography. *(b)* Western blotting was performed on cold translation reactions with MAb 6C1 and the polyclonal anti-MAGE-10 rabbit serum. Lysate of MZ2-MEL-3.0 cells was electrophoresed along with the translation products for comparison. The band of 46 kDa in MZ2-MEL-3.0 cells is MAGE-1.

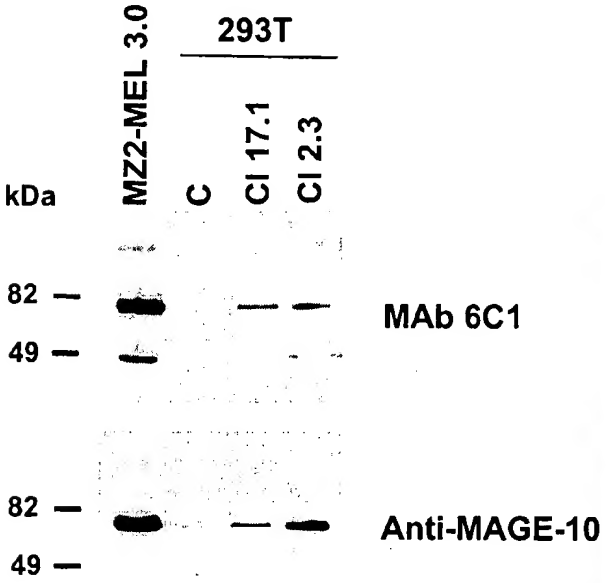

FIGURE 3 – Transient transfection of a *MAGE-10* cDNA clone. Human 293T cells were transfected with 2 independent *MAGE-10* cDNA clones (Cl 17.1 and 2.3). Mock transfection (C) was performed as a negative control. Cell lysates were prepared 48 hr later and analyzed by Western blotting. Duplicate blots were analyzed with MAb 6C1 and polyclonal anti-MAGE-10 antibody. Lysate of MZ2-MEL-3.0 cells was electrophoresed along with the samples as a positive control.

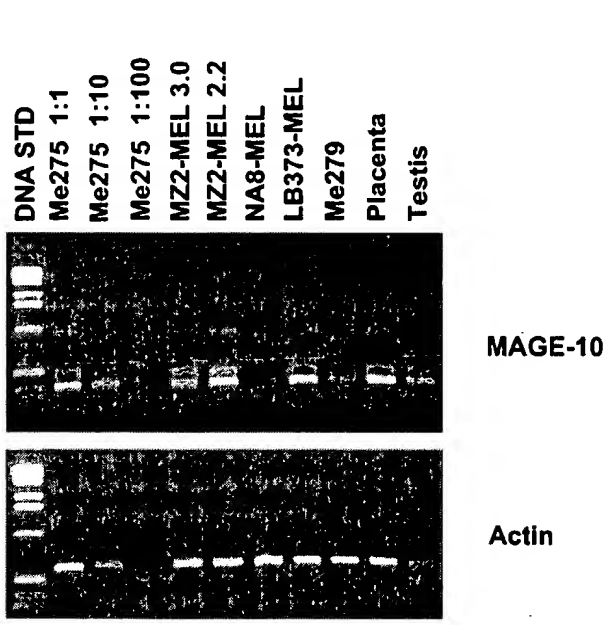

FIGURE 4 – Analysis of MAGE-10 mRNA expression by RT-PCR. cDNA from the indicated human melanoma cell lines and tissues was synthesized, and aliquots were used as templates for amplification with *MAGE-10* and actin-specific primers as described in Material and Methods. To allow semi-quantitative evaluation of results, serial 1:10 dilutions of RNA from the melanoma cell line Me275 were used for cDNA synthesis. Standard DNA is a 1-kb ladder.

probe, a 485-bp Hpa I fragment from the *MAGE-10* cDNA insert isolated from phage 13.1 was labeled with ^{32}P using a random-primed DNA-labeling kit (Boehringer Mannheim). After hybridization, the membrane was washed under stringent conditions. The RNA marker was from Promega (Zurich, Switzerland).

Purification of villous cytotrophoblast cells

Cytotrophoblast cells were purified from term placenta obtained immediately after cesarean section. Enzymatic digestion followed by Percoll gradient centrifugation and establishment of primary cultures were performed as described (Guillaudeux *et al.*, 1995).

RESULTS

Cloning of a MAGE-10 *cDNA*

To identify the gene encoding the 72-kDa protein recognized by the anti-MAGE-1 MAb 6C1, an expression library prepared from MZ2-MEL-43 melanoma cells was screened with the antibody. Partial sequencing of the cDNA inserts of positive plaques identified sequences corresponding to members of the *MAGE-A* gene family. The strongest signals were obtained with phage clones containing *MAGE-1* and, surprisingly, *MAGE-10* cDNAs. Sequencing of the entire 1.4 kb *MAGE-10* insert from 1 of 3 identical clones confirmed that it was identical to the 3' end of the published *MAGE-10* genomic sequence with an additional extension of 660 bp at the 3' end.

To obtain a full-length *MAGE-10* cDNA, a 485-bp Hpa I fragment from the 1.4-kb cDNA was used to screen 2 independent cDNA libraries from melanoma cells. Two types of clone were identified, approximately 2.5 and 1.5 kb long. The latter was derived from an alternative oligo-dT annealing to an internal poly-A stretch located 77 bp downstream of the coding region. Sequencing of individual clones and alignment to the known *MAGE-10* genomic sequence allowed delineation of the intron–exon structure of the gene (Fig. 1). *MAGE-10* contains 4 exons, the last 2 corresponding to those predicted by De Plaen *et al.* (1994) by analogy to the previously characterized *MAGE* genes. The sequence confirms the presence in the last exon of an open reading frame encoding a protein of 369 amino acids. Exon 4 of *MAGE-10* differs from the last exons of the other *MAGE-A* genes by the presence of a longer 3'-untranslated region, resulting in a cDNA that is 0.7 kb longer.

As exon 1 was not included in the known genomic sequence, a *MAGE-10*-containing cosmid was used to amplify the region spanning exons 1 and 2. The first intron is 1,498 nucleotides long. Remarkably, exons 1 and 2 showed the highest homology to sequences located in introns of *MAGE-A* genes, particularly *MAGE-4a* and *-4b*. Exon 2 of *MAGE-10* was also found to be partially homologous to the 2nd exon of *MAGE-2*. *MAGE-2* and *-10* are so far the only members of the *MAGE-A* gene family known to contain 4 exons.

MAGE-10 *cDNA encodes the 72-kDa protein recognized by anti-MAGE-1 MAb*

When *MAGE-10* cDNA clones were transcribed and translated *in vitro* in the presence of ^{35}S-methionine, the reaction yielded a major radiolabeled product of approximately 72 kDa (Fig. 2*a*). In addition, duplicate cold reactions were set up in parallel and analyzed by Western blotting with the anti-MAGE-1 MAb 6C1. As shown in Figure 2*b* (left panel), the MAb specifically recognized the 72-kDa translated product. The same product was also recognized by a polyclonal anti-MAGE-10 serum (Fig. 2*b*, right panel). This serum was obtained after immunization with a peptide derived from the carboxy terminus of MAGE-10, a sequence that shares no homology with other known MAGE proteins. Finally, *MAGE-10* cDNAs were transiently transfected into human 293T cells. Western blot analyses of lysates of transfected cells with MAb 6C1 and the anti-MAGE-10 polyclonal serum identified a protein of 72 kDa similar to that detected in lysates of melanoma cells MZ2-MEL-3.0 (Fig. 3).

MAGE-10 mRNA expression in normal and tumor tissues

Expression of *MAGE-10* was studied in various tissues by RT-PCR amplification. A pair of specific oligonucleotide primers, located in exons 2 and 4, were chosen to distinguish amplified cDNA from contaminant genomic DNA. Figure 4 shows a representative RT-PCR analysis of melanoma cell lines and tissues. In addition to the predicted 410-bp product, a less intense band of approximately 500 bp was co-amplified in all positive samples. Sequencing of the 2 amplification products confirmed that they were identical to *MAGE-10* and revealed the existence of an alternatively or incompletely spliced form of mRNA giving rise to the longer PCR product. This alternative mRNA species contained an extra 74-bp exon (designated exon 2a in Fig. 1) and appeared to represent a minor fraction of *MAGE-10* transcripts, as indicated by the fact that none of the 22 cDNA clones isolated contained the additional exon. Analysis of a series of fresh melanoma tumors showed that *MAGE-10* is usually co-expressed with *MAGE-1* (Table I), confirming the results obtained by Western blotting with melanoma cell lines (Carrel *et al.*, 1996). In contrast, all samples of metastatic ocular melanoma and breast cancer (11 samples each) were negative. Only 1 in 10 glioblastoma tumors was positive for MAGE-10 mRNA (not shown), and this tumor also expressed MAGE-1.

MAGE-10 transcripts were further analyzed by Northern blotting in melanoma tumors and cell lines. A band of approximately 2.5 kb was detected in tumor LAU-202 and cell lines LB373-MEL-4.0 and MZ2-MEL-3.0 but not in tumor LAU-165 or NA8-MEL

TABLE I – EXPRESSION OF *MAGE-10* RT USING PCR

Sample	*MAGE-10*	Sample	*MAGE-10*	*MAGE-1*
Normal tissues, adult		*Melanoma (cutaneous)*		
Testis	++[1]	LAU-99	−	+
Placenta	+++	LAU-194	+++	+++
Colon	−	LAU-36	++	++
Lung	−	LAU-214	++	+++
Kidney	−	LAU-169	+++	+++
Spleen	−	LAU-177	++	+++
Brain	−	LAU-195	++	+
Brain (telencephalum)	−	LAU-202	+++	+++
Brain (cortex)	−	LAU-193	++	+++
Brain (cerebellum)	−	GE-RC	++	+++
Mammary gland	−	LAU-101	+	−/+
Adrenal gland	−	LAU-119	++	++
Heart	−	LAU-6	+++	++
Uterus (endometrium)	−	LAU-148	++	++
Ovary	−	LAU-50	+++	++
Liver	−	LAU-165	−	−
Bladder	−	LAU-162	−	−
Small intestine	−	SW-641118	−	−
Prostate	−	LAU-132	−	−
Umbilical cord	−	LAU-142	−/+	−
Skin	−	LAU-147	−	−
Scar tissue	−	LAU-53	++	−/+
Melanocytes	−	LAU-117	−	−
		LAU-56	−	−
Fetal tissues		LAU-4	−	−
Liver	−	LAU-90	−	−
Brain (cerebellum)	−	LAU-91	−	−/+
Brain (cortex)	−	LAU-205	−	−
Spleen	−	LAU-18	−	−
Thymus	−			
Meninges	−			

[1]Levels of expression were analyzed semi-quantitatively by comparison with serial RNA dilutions of cell line Me275. Scoring was as follows: +++, >50%; ++, 10% to 50%; and +, 1% to 10% of the level in the reference line. −/+, faintly visible band.

cells (Fig. 5), in agreement with the RT-PCR results (Table I, Fig. 4). The level of mRNA detected in melanoma appeared to be similar to that found in placenta.

A series of healthy adult tissues, including scar tissue, and some fetal tissue samples were also subjected to RT-PCR. As for the other members of the *MAGE* gene family, MAGE-10 mRNA was expressed only in testis and placenta (Table I, Fig. 4).

Detection of MAGE-10 protein in human placenta

Of the 2 normal tissues expressing MAGE-10, testis and placenta, the latter appeared to express MAGE-10 at relatively high levels (Figs. 4, 5). We thus analyzed MAGE-10 by Western blot in lysates of fresh placental tissue and villous trophoblast cells cultured *in vitro*. Cytotrophoblasts from term placenta are known to undergo spontaneous functional and morphological differentiation when cultured *in vitro* for 72 to 96 hr. Abundant levels of MAGE-10 are detected with both MAb 6C1 and the polyclonal anti-MAGE-10 serum in trophoblast cells cultured for either 24 or 72 hr (Fig. 6). These preparations are enriched in cyto- and syncytio-trophoblasts, respectively, as confirmed by the expression of chorionic gonadotropin (Fig. 6, lower panel). The amount of MAGE-10 protein expressed in trophoblasts is similar to that found in melanoma cells MZ2-MEL-3.0. As expected from PCR results (De Plaen *et al.*, 1994), MAGE-1 was not detected in placenta by MAb 6C1, while the weak band migrating above MAGE-1 indicates the presence of MAGE-3 and/or -4.

MAGE-10 is a nuclear protein

To determine the subcellular localization of MAGE-10, immunofluorescence analysis was performed on MZ2-MEL-3.0 cells, which express several *MAGE* genes, including *MAGE-1* and -10. Strong nuclear staining was obtained with the anti-MAGE-10 serum but not with control pre-immune serum (Fig. 7*a*). MAGE-10 appeared to be uniformly distributed in the nucleus, except for nucleoli, which appeared to be excluded. No staining was observed in *MAGE-10*-negative cell lines using the polyclonal serum (not shown). Staining of MZ2-MEL-3.0 cells with MAb 6C1 (recognizing both MAGE-1 and 10) showed prominent nuclear staining plus weaker diffuse cytoplasmic staining. The nuclear localization of MAGE-10 in melanoma cells was confirmed by transfection of a *MAGE-10* cDNA into MAGE-negative NA8-MEL melanoma cells. While MAGE-1 localizes mainly in the cytoplasm, MAGE-10 is present in the nucleus of transfected cells (Fig. 7*b*).

DISCUSSION

MAGE-10 has an unexpected electrophoretic mobility by SDS-PAGE under reducing conditions. Its apparent m.w. is 72 kDa, whereas that of other MAGE proteins ranges from 45 to 50 kDa (Kocher *et al.*, 1995; Amar-Costesec *et al.*, 1994; Shichijo *et al.*,

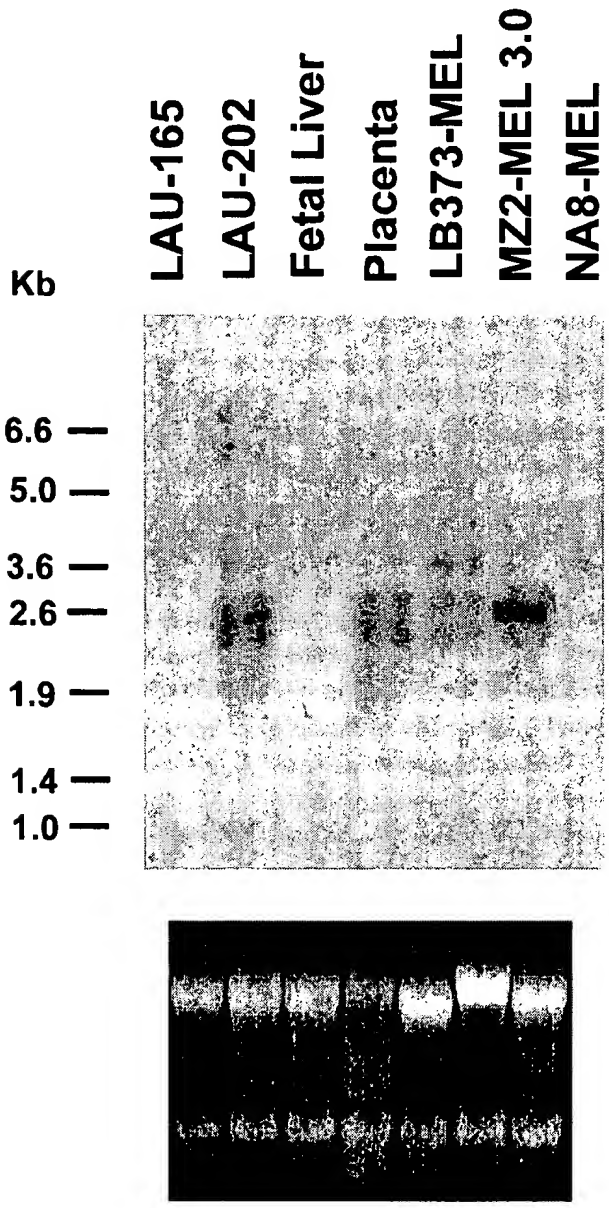

FIGURE 5 – Northern blot analysis of MAGE-10 transcripts. RNA was extracted from melanoma tumors LAU-165 and -202, normal human tissue and melanoma cell lines LB373-MEL, MZ2-MEL-3.0 and NA8-MEL. Aliquots (20 μg) were electrophoresed, transferred to a nylon membrane and hybridized with a ^{32}P-labeled *MAGE-10* cDNA probe. Numbers indicate the electrophoretic mobility of RNA size markers. Bottom panel shows ethidium bromide staining of the gel.

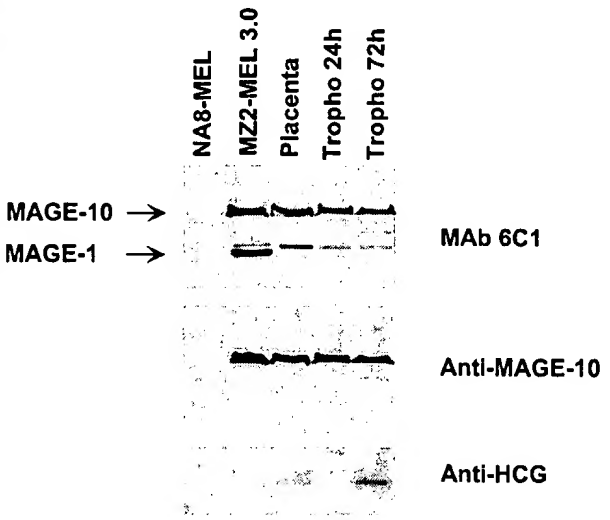

FIGURE 6 – Expression of MAGE-10 in placenta. Western blot analysis was performed with lysates of fresh placental tissue, and purified trophoblast cells were cultured *in vitro* for 24 and 72 hr (consisting primarily of cyto- and syncytio-trophoblast, respectively). Duplicate blots were incubated with MAb 6C1 and the polyclonal anti-MAGE-10 serum. After stripping, one blot was used to detect chorionic gonadotropin β (HCG).

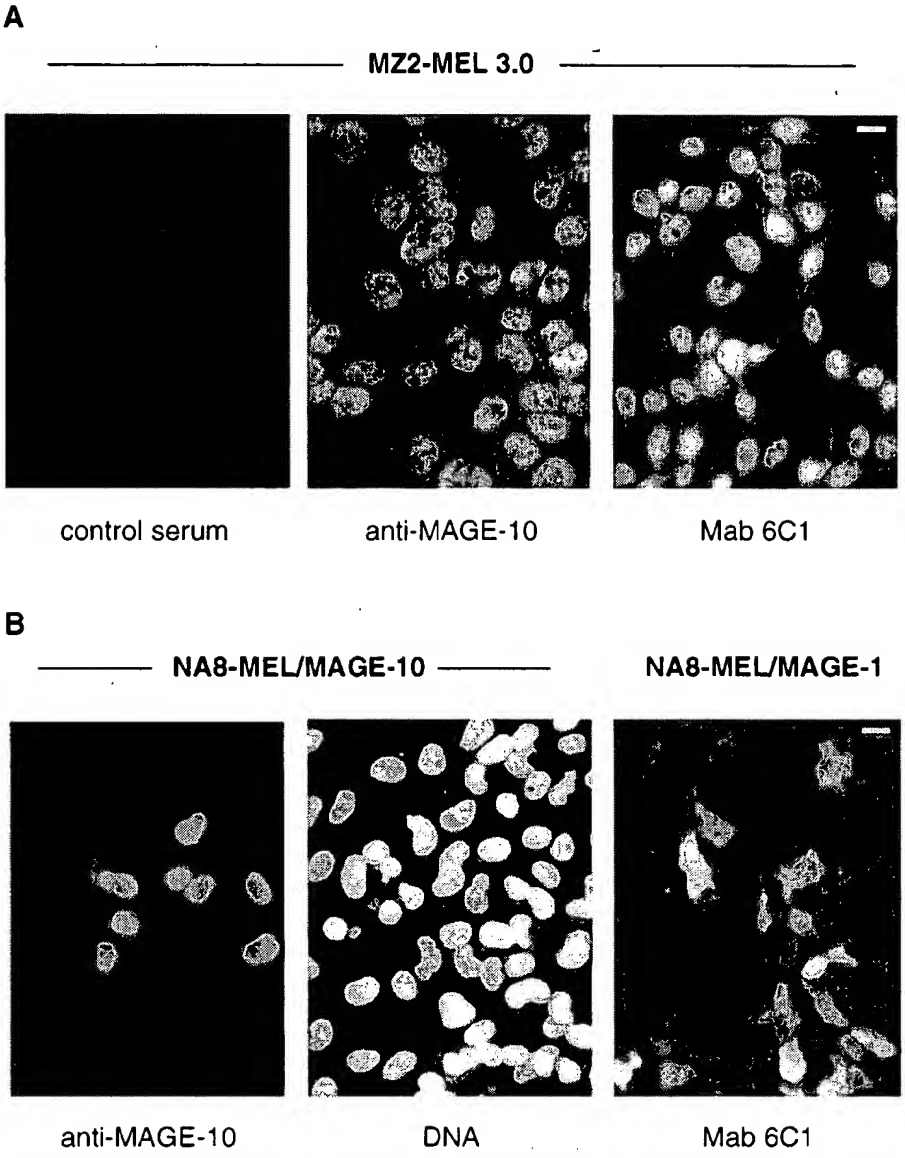

FIGURE 7 – Immunofluorescence staining of melanoma cells with anti-MAGE-10 antibodies. Melanoma cells were grown in chamber slides, fixed and stained with MAb 6C1, control pre-immune or anti-MAGE-10 serum. *(a)* MZ2-MEL-3.0 (MAGE-1- and -10-positive). *(b)* NA8-MEL cells transiently transfected with MAGE-10 and MAGE-1 cDNAs. Nuclei were visualized by staining chromosomal DNA. Scale bar = 10 μm.

1995; Jurk *et al.*, 1998). Although the putative MAGE-10 protein is only 50 amino acids longer than other proteins of the *MAGE-A* family, other explanations must be sought. Post-translational modifications could be involved, but so far we have no evidence that these occur, as preliminary pulse-chase experiments suggest that the primary translation product migrates like the mature form. Abnormally slow migration in PAGE has been described for other proteins and sometimes attributed to a very high content in charged amino acids. However, this is not the case for MAGE-10.

The nuclear localization of MAGE-10 is an interesting feature. While MAGE-1 and -3 have been reported to be cytoplasmic proteins (Kocher *et al.*, 1995; Amar-Costesec *et al.*, 1994), MAGE-11 has been shown to be localized predominantly in the nucleus of HeLa cells (Jurk *et al.*, 1998). Necdin, a mouse protein displaying high homology with the MAGE family and implicated in the growth arrest of post-mitotic neurons, is also localized in the nucleus (Hayashi *et al.*, 1995). The sequence responsible for the nuclear targeting in MAGE-10, -11 and necdin remains to be determined. MAGE-10 contains a nuclear localization signal of the SV40 large T antigen type (amino acid residues 5 to 11); however, this type of sequence is not present in MAGE-11 or necdin. Interestingly, MAGE-10 is predicted to be a nuclear protein based on its amino acid composition, according to Reinhardt's neural network (Reinhardt and Hubbard, 1998). Expression of truncated versions of MAGE-10 should clarify whether a specific sequence is responsible for nuclear targeting.

An intriguing aspect of the *MAGE* gene families is that, while a great deal is known about the expression of these genes in different malignancies, their function in normal tissue is unknown. Murine *MAGE* homologs, named *SMAGE*, have been isolated (De Backer

et al., 1995). Like *MAGE*, they are expressed in testis. Interestingly, specific transcripts were detected in embryos. These findings together with the demonstration that some members of the MAGE-A protein family are located in the nucleus (this report and Jurk *et al.*, 1998) suggest that these proteins may be involved in cell division processes during embryogenesis. The hypothesis that MAGE-10 regulates cell division is currently under study, as is the search for proteins that potentially interact with MAGE-10.

MAGE genes represent interesting candidates for specific immunotherapy of cancer. Using the anti-MAGE-1/MAGE-10 MAb 6C1, we had previously found that MAGE-10 is expressed in several melanoma cell lines. Here, RT-PCR analyses confirm the expression of *MAGE-10* in samples of cutaneous melanoma. Approximately 50% of metastatic lesions express MAGE-10, and most of these tumors also express MAGE-1. In contrast, as already observed for *MAGE-1, -2, -3* and *-4* (Mulcahy *et al.*, 1996), we did not detect *MAGE-10* expression in ocular melanomas. Northern and Western blot analyses show that *MAGE-10* is expressed quite abundantly in melanoma tumors, at both the RNA and protein levels. In particular, MAGE-10 protein appears to be at least as abundant as MAGE-1. Previous results, based solely on RT-PCR, indicated that tumors expressed MAGE-10 only at very low levels (De Plaen *et al.*, 1994). Different technical reasons, such as suboptimal RT-PCR conditions, could explain this discrepancy. In the present study, cDNA cloning allowed the design of better oligonucleotide primers. Isolation of *MAGE-10* cDNA has also revealed some interesting features. MAGE-10 mRNA, which has a size of approximately 2.5 kb, has a longer 3'-untranslated region compared with the other *MAGE-A* transcripts characterized to date, which are 1.8 kb long. Unlike the majority of Xq28 *MAGE-A* genes, which contain 3 exons, *MAGE-10* contains 4 exons and, thus, in this respect resembles *MAGE-2*. Like *MAGE-2*, exon 2 of *MAGE-10* shares the highest homology to intron sequences of other *MAGE* genes. cDNA isolation of the remaining uncharacterized family members should allow a better comparison of the relative structure of these genes.

Specific CTL epitopes have been identified in MAGE-1, -2 and -3 proteins. As for the *BAGE* and *GAGE* gene families, a particularly attractive characteristic is their tumor-restricted pattern of expression (Van den Eynde and Boon, 1997). Testis and, for *MAGE-3, -4, -8, -9, -10* and *-11*, placenta are the only normal adult tissues to express these families of genes. In testis, expression of *MAGE-1* and *-4* has been localized to spermatogonia and primary spermatocytes, which do not express detectable HLA class I molecules (Takahashi *et al.*, 1995; Janitz *et al.*, 1994). We have observed a similar localization using our anti-MAGE-1/MAGE-10 MAb 6C1 (data not shown); thus, these cells would not be at risk during a MAGE-specific immunization protocol. In this report, using MAb 6C1 and the MAGE-10-specific polyclonal antibody, we have shown that abundant levels of MAGE-10 are present in purified trophoblast cells. Like gametogenic cells, the latter are largely devoid of classical HLA molecules at the surface (Hammer *et al.*, 1997). Since several potential CTL epitopes, as predicted by searching for consensus HLA-binding motifs are present in MAGE-10 and the protein appears to be expressed in tumors at abundant levels, it is worth pursuing the search for MAGE-10-derived immunogenic peptides.

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